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# Overexpression of CupB5 activates alginate overproduction in *Pseudomonas aeruginosa* by a novel AlgW-dependent mechanism

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## Summary

In *P. aeruginosa*, alginate overproduction, also known as mucoidy, is negatively regulated by the transmembrane protein MucA, which sequesters the alternative sigma factor AlgU. MucA is degraded via a proteolysis pathway that frees AlgU from sequestration, activating alginate biosynthesis. Initiation of this pathway normally requires two signals: peptide sequences in unassembled outer-membrane proteins (OMPs) activate the AlgW protease, and unassembled lipopolysaccharides bind periplasmic MucB, releasing MucA and facilitating its proteolysis by activated AlgW. To search for novel alginate regulators, we screened a transposon library in the non-mucoid reference strain PAO1, and identified a mutant that confers mucoidy through overexpression of a protein encoded by the *chaperone-ushe* pathway gene *cupB5*. CupB5-dependent mucoidy occurs through the AlgU pathway and can be reversed by overexpression of MucA or MucB. In the presence of activating OMP peptides, peptides corresponding to a region of CupB5 needed for

mucoidy further stimulated AlgW cleavage of MucA *in vitro*. Moreover, the CupB5 peptide allowed OMP-activated AlgW cleavage of MucA in the presence of the MucB inhibitor. These results support a novel mechanism for conversion to mucoidy in which the proteolytic activity of AlgW and its ability to compete with MucB for MucA is mediated by independent peptide signals.

**Keywords:** *Pseudomonas aeruginosa*, alginate, MucA, MucB, CupB5, signal transduction

## Introduction

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*Pseudomonas aeruginosa* is an important opportunistic human pathogen, capable of thriving in different environmental niches. Activation of the AlgU-regulated extracytoplasmic stress response results in the production of copious amounts of an exopolysaccharide called alginate, forming a capsule that protects the bacterium from the host-immune system. Alginate production by *P. aeruginosa* is a particular problem for patients with cystic fibrosis, whose dehydrated mucus-filled lungs provide an environment especially suited for bacterial infection ([Folkesson et al., 2012](#)). Nonmucoid strains initially colonize the lungs, and alginate overproduction caused by mutations in *mucA* results in a mucoid phenotype and a chronic lung infection ([Ciofu et al., 2005](#); [Hogardt et al., 2007](#); [Mena et al., 2008](#)). Among many roles in pathogenesis, alginate can promote adherence to lung epithelial cells, reduce the efficiency of phagocytosis, and protect bacteria from antibiotics ([Govan and Deretic, 1996](#); [Pier et al., 2001](#); [Leid et al., 2005](#)).

The extracytoplasmic stress-response pathways in *P. aeruginosa*, *Escherichia coli*, and other Gram-negative bacteria involve regulated intramembrane proteolysis and share common features. For example, outer-membrane and periplasmic stress initiates a proteolytic cascade that ultimately degrades a transmembrane anti-sigma factor and releases the sigma factor to up-regulate stress-response genes ([Alba et al., 2002](#); [Kanehara et al., 2002](#); [Walsh et al., 2003](#); [Akiyama et al., 2004](#); [Flynn et al., 2004](#); [Chaba et al., 2007](#)). In *P. aeruginosa*, proteolysis of wild type MucA, the anti-sigma factor, releases the AlgU sigma factor, which enhances transcription of the genes responsible for alginate biosynthesis from the *algD* promoter ([Martin et al., 1993](#); [Wood et al., 2006](#)).

In *P. aeruginosa*, degradation of MucA is normally initiated by cleavage by the AlgW protease at site 1 near the periplasmic face of the inner membrane, continues with cleavage by the MucP protease at site 2 near the cytoplasmic face of the membrane, and is completed in the cytoplasm by AAA+ proteases such as ClpXP ([Akiyama et al., 2004](#); [Alba et al., 2002](#); [Chaba et al., 2007](#); [Flynn et al., 2004](#); [Kanehara et al., 2002](#); [Qiu et al., 2007](#); [Qiu et al., 2008b](#); [Walsh et al., 2003](#)). Two signals are required to initiate this proteolytic cascade. The C-terminal peptides of proteins that accumulate in the periplasm during stress, including outer-membrane proteins (OMPs), bind to the PDZ domains of the trimeric AlgW protease and allosterically activate site-1 cleavage of MucA, but MucB competes for MucA binding and can inhibit this cleavage ([Cezairliyan and Sauer, 2009](#)). Recent studies show that lipopolysaccharides (LPS), a key component of the outer membrane, can bind MucB and cause release of MucA ([Lima et al., 2013](#)).

Here, we identify CupB5 as a novel activator of the alginate-production pathway. CupB5, an adhesive protein, is a member of one of the chaperone/usher pathways (CupA-E) that mediate assembly of pili and play roles in bacterial pathogenesis, adhesion, and biofilm formation ([Hull et al., 1981](#); [Marklund et al., 1992](#); [Soto and Hultgren, 1999](#); [Vallet et al., 2001](#); [Giraud et al., 2011](#); [Mikkelsen et al., 2009](#)). These secretion systems, which are best characterized in Gram-negative bacteria ([Sauer et al., 2000](#); [Waksman and Hultgren, 2009](#)), contain a periplasmic chaperone that maintains proteins to be secreted in an unassembled state and an usher that transports these proteins through the outer membrane. We find that overexpression of CupB5 in the absence of its chaperone induces a mucoid phenotype in *P. aeruginosa* and provide evidence that a peptide signal in CupB5 enhances OMP-activated cleavage of MucA by AlgW, even in the presence of MucB, allowing initiation of AlgU signaling.

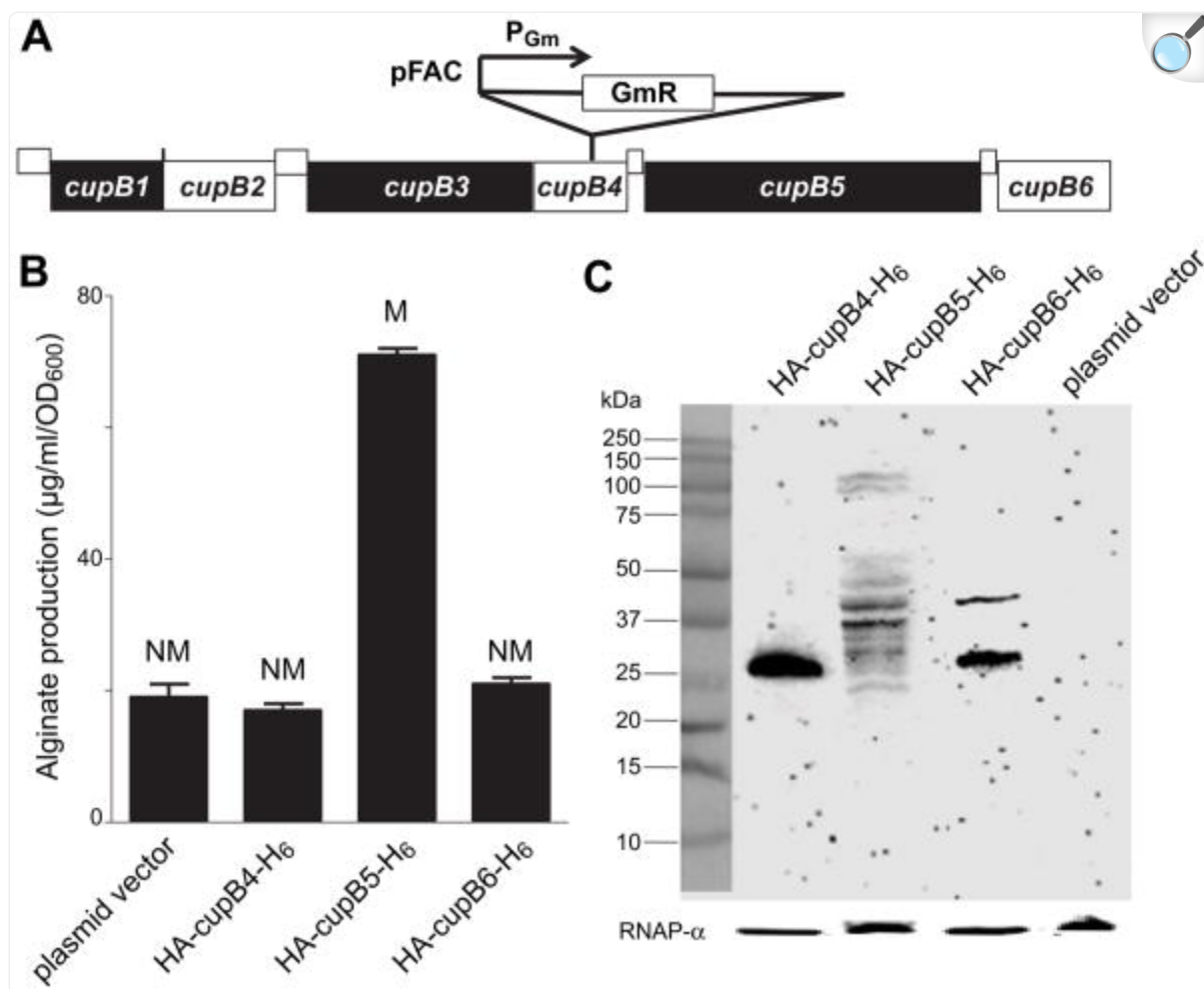
## Results

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### Overexpression of CupB5 induces mucoidy

To identify new alginate regulatory genes, we performed mariner-transposon mutagenesis ([Wong and Mekalanos, 2000](#)) in the nonmucoid *P. aeruginosa* reference strain PAO1. We isolated a mucoid variant (PAO1-VE22) and identified the site of a single-copy insertion by inverse PCR and Southern-blot analysis (data not shown). The transposon insertion was six base pairs before the TAG stop codon at the 3' end of *cupB4* ([Fig. 1A](#)) and introduced a  $\sigma^{70}$ -dependent  $P_{Gm}$  promoter that drove expression of the gentamicin resistance cassette and downstream genes ([Rubin et al., 1999](#)). Alginate production by PAO1-VE22 ( $31 \pm 1.5$   $\mu\text{g/ml/OD}_{600}$ ) was substantially higher than that of PAO1 ( $8.5 \pm 0.02$   $\mu\text{g/ml/OD}_{600}$ ) after 24 h growth at 37°C. To test if altered expression of *cupB4*, *cupB5*, or *cupB6* was responsible for the mucoidy of PAO1-VE22, we cloned each individual coding sequence with an N-terminal HA epitope and C-terminal H<sub>6</sub> tag in pHERD20T under control of an arabinose-inducible promoter ([Qiu et al., 2008a](#)). Plasmids expressing the tagged variant of CupB5, but not of CupB4 or CupB6, induced mucoid conversion and high-level alginate production in PAO1 ([Fig. 1B](#)). Western-blot analysis indicated that the plasmid-expressed CupB4, CupB5, and CupB6 variants were detectable when overexpressed in PAO1, albeit with fragmentation of CupB5 ([Fig. 1C](#)). We conclude that enhanced expression of CupB5 or a CupB5 fragment induces mucoidy and increased alginate in PAO1-VE22.

Fig. 1. Overproduction of CupB5 causes mucoid conversion in PAO1.



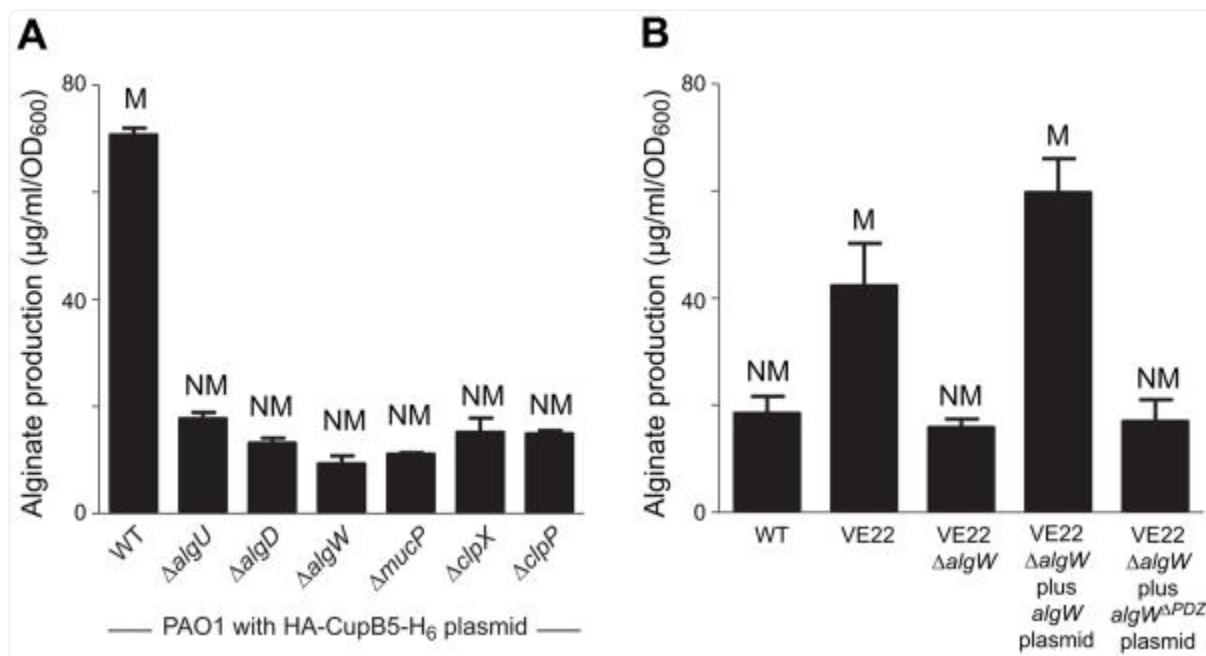
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(A) Organization of the *cupB1-cupB6* gene cluster ([www.pseudomonas.com](http://www.pseudomonas.com)), and location of the transposon insertion site in PAO1-VE22. (B) Alginate production, measured using the carbazole assay, and colony phenotypes (M, mucoid; NM, nonmucoid) were assayed following transformation of *P. aeruginosa* strain PAO1 with plasmid HERD20T or variants expressing HA-CupB4-H<sub>6</sub>, HA-CupB5-H<sub>6</sub>, or HA-CupB6-H<sub>6</sub>, and overnight growth of colonies on PIA plates (carbenecillin 300  $\mu\text{g ml}^{-1}$ , 0.1% L-arabinose) at 37°C. Values are averages  $\pm$  SEM (n=3). (C) Cell lysates of PAO1 carrying appropriate plasmids were electrophoresed on SDS polyacrylamide gels, and Western-blots using an anti-HA monoclonal antibody were used to detect HA-CupB4-H<sub>6</sub>, HA-CupB5-H<sub>6</sub>, or HA-CupB6-H<sub>6</sub>. The rightmost lane shows that HA cross-reactive proteins were absent in PAO1 transformed with the pHERD20T empty-vector.

## Genetic requirements for CupB5-induced mucoidy

Deletion of five single genes required for the AlgU response (*algW*, *mucP*, *clpX*, *clpP*, or *algU*) in PAO1 prevented mucoidy and high-level alginate production when HA-CupB5-H<sub>6</sub> was overproduced from a plasmid in these strains ([Fig. 2A](#)). In-frame deletion of *algW* in PAO1-VE22 also prevented alginate overproduction and mucoidy, a phenotype that was complemented by plasmid expression of AlgW but not AlgW lacking its PDZ domain ([Fig. 2B](#)). Consistently, when AlgU-dependent promoters were fused to *lacZ* and introduced into PAO1-VE22 or PAO1-VE2, a strain in which AlgW is activated by overproduction of MucE ([Qiu et al., 2007](#)),  $\beta$ -galactosidase levels were increased markedly compared to the same promoter fusions in PAO1 ([Fig. S1](#)). CupB5 is normally an extracellular protein ([Ruer et al., 2008](#); [Vallet et al., 2001](#)). However, its overexpression in the absence of corresponding levels of the CupB4 chaperone and usher could result in periplasmic accumulation that acts directly or indirectly through AlgW to activate alginate production.

Fig. 2. Genetic requirements for CupB5-mediated alginate overproduction.



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(A) Wild-type (WT) or mutant PAO1 strains were transformed with pHERD20 encoding HA-CupB5-H<sub>6</sub>, grown on PIA plates with 0.1% L-arabinose for 24 h at 37°C to induce HA-CupB5-H<sub>6</sub> overexpression, and mucoid phenotypes and alginate production were determined. The *algD* gene encodes an enzyme required for alginate production. The *algU*, *algW*, *mucP*, *clpX*, and *clpP* genes are required for induction of *algU*-dependent alginate production. (B) Alginate production and mucoid phenotypes of different strains were measured after growth for 24 h at 37°C on PIA plates plus 300 µg m<sup>-1</sup> carbenecillin and 0.1% L-Ara. In both panels, values are averages ± SEM (n=3).

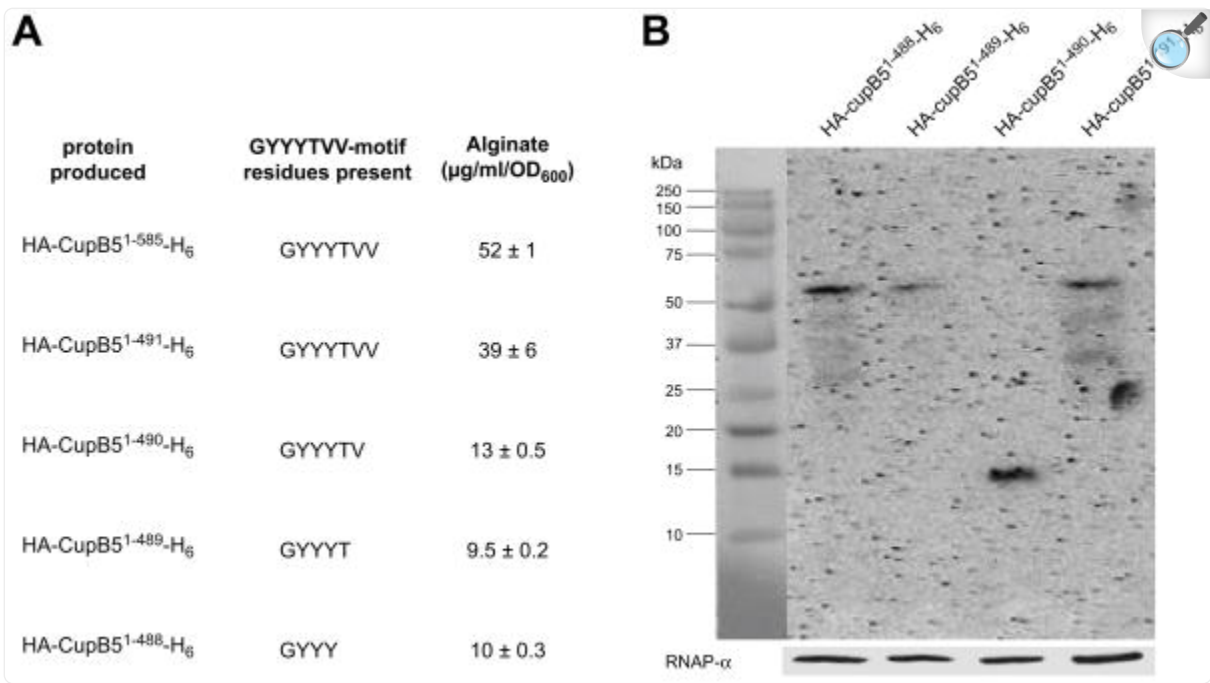
## CupB5 contains an internal signal for alginate overproduction

Because C-terminal sequences terminating with an aromatic residue and α-carboxylate can activate AlgW, we initially tested if the C-terminal residues of CupB5 (Asn<sup>1016</sup>-Ile<sup>1017</sup>-Trp<sup>1018</sup>) were responsible for activating AlgW and inducing mucoidy. However, overexpression of CupB5 variants with a C-terminal H<sub>6</sub> tag or containing a deletion of the Asn<sup>1016</sup>-Ile<sup>1017</sup>-Trp<sup>1018</sup> sequence and a C-terminal H<sub>6</sub> tag still induced mucoidy in PAO1 (Table S1). Thus, the extreme C-terminal residues of CupB5 play little or no role in mediating alginate overproduction. Moreover, overproduction of variants containing just the N-terminal 491 residues of CupB5 also induced mucoidy (Table S1), indicating that a major

inducing signal is located in this portion of the protein.

Deletion analyses revealed that the Thr<sup>489</sup>-Val<sup>490</sup>-Val<sup>491</sup> sequence of CupB5 appeared important for alginate overproduction. Specifically, overexpression of CupB5<sup>1-491</sup> produced substantially more alginate than overexpression of CupB5<sup>1-489</sup> or CupB5<sup>1-488</sup> (Fig. 3A), and Western blotting showed that these proteins were present at a similar intracellular levels (Fig. 3B). Cells expressing CupB5<sup>1-490</sup> also produced less alginate (Fig. 3A), but this protein appeared to be severely truncated by degradation (Fig. 3B). Based on these results, we hypothesized that the CupB5 activating signal included some or all of the Thr<sup>489</sup>-Val<sup>490</sup>-Val<sup>491</sup> sequence.

Fig. 3. Delineation of the CupB5 signal that activates alginate production.



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(A) CupB5 variants with C-terminal truncations were cloned into pHERD20T, expressed in strain PAO1, and alginate production following growth in the presence of 1% L-arabinose was assayed. Proteins containing CupB5 residues 1–585 and 1–491 contain the complete GYYYTVV<sup>491</sup> sequence motif. The remaining proteins contain only a portion of this motif. (B) Western-blot detection using anti-HA monoclonal antibody of truncated CupB5 variants.

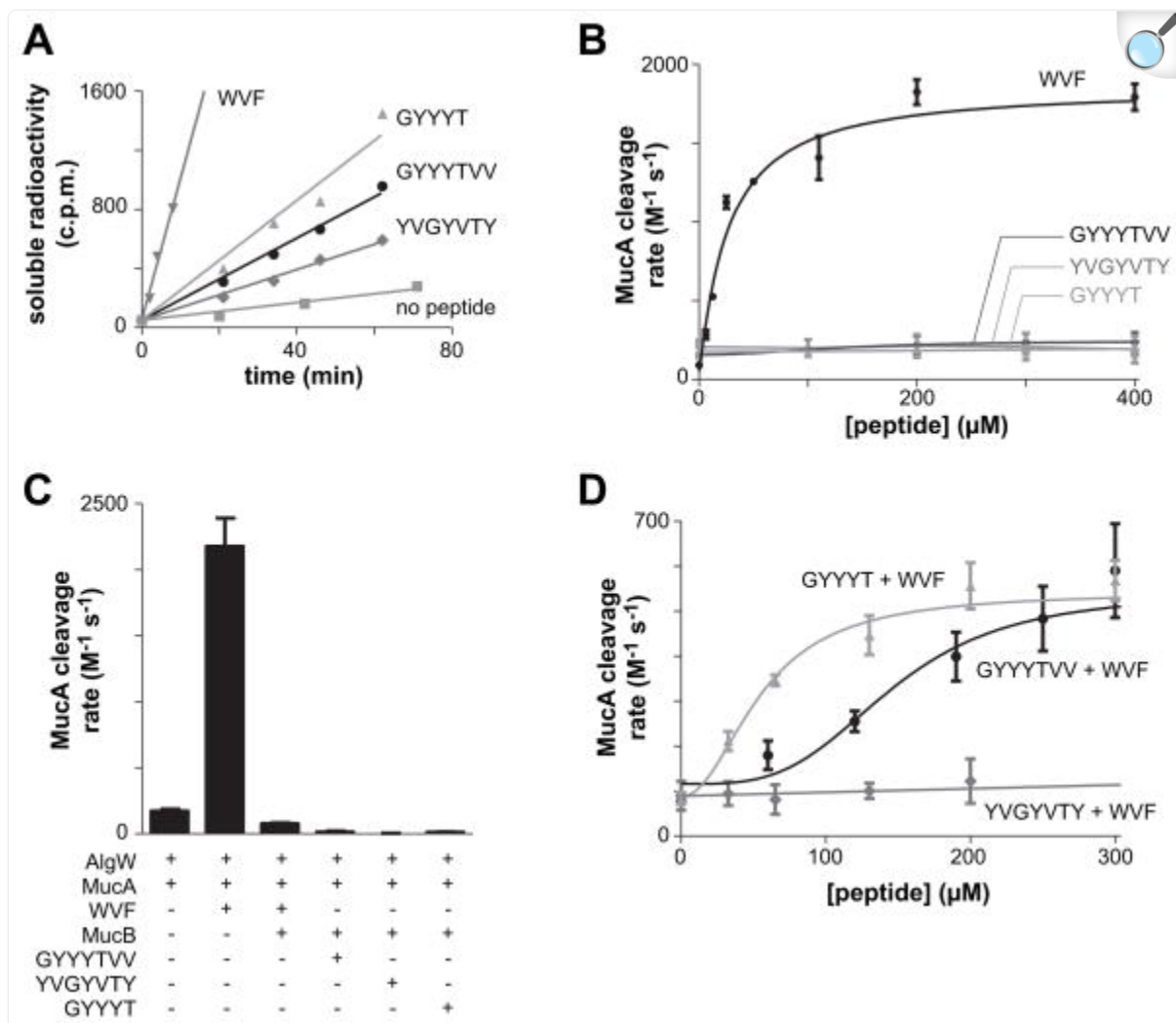
CupB5 peptides stimulate OMP-activated AlgW cleavage of MucA and partially relieve

## MucB inhibition *in vitro*

We initially tested if synthetic peptides including the Thr<sup>489</sup>-Val<sup>490</sup>-Val<sup>491</sup> CupB5 sequence might stimulate alginate production in a manner analogous to OMPs by activating AlgW cleavage of <sup>35</sup>S-MucA. One peptide (GYYYTVV) corresponded to the seven C-terminal residues of CupB5<sup>1-491</sup>, another (YVG YVTY) was a sequence-scrambled variant, and a third (GYYYT) corresponded to the C-terminus of CupB5<sup>1-489</sup>, which did not stimulate alginate production *in vivo*. We assayed the kinetics of AlgW cleavage of <sup>35</sup>S-MucA *in vitro* by the production of acid-soluble peptide fragments in the presence of different concentrations of the CupB5 peptides. Each CupB5 peptide stimulated AlgW cleavage of <sup>35</sup>S-MucA to a small degree, but at far lower rates than a peptide (WVF) corresponding to the C-terminal residues of MucE, a potent activator of AlgW ([Fig. 4A & 4B](#)). Thus, the CupB5 peptides do not substitute for OMP-like peptides in robust activation of AlgW cleavage of MucA.



Fig. 4. CupB5 peptides relieve MucB inhibition of MucA cleavage by AlgW.



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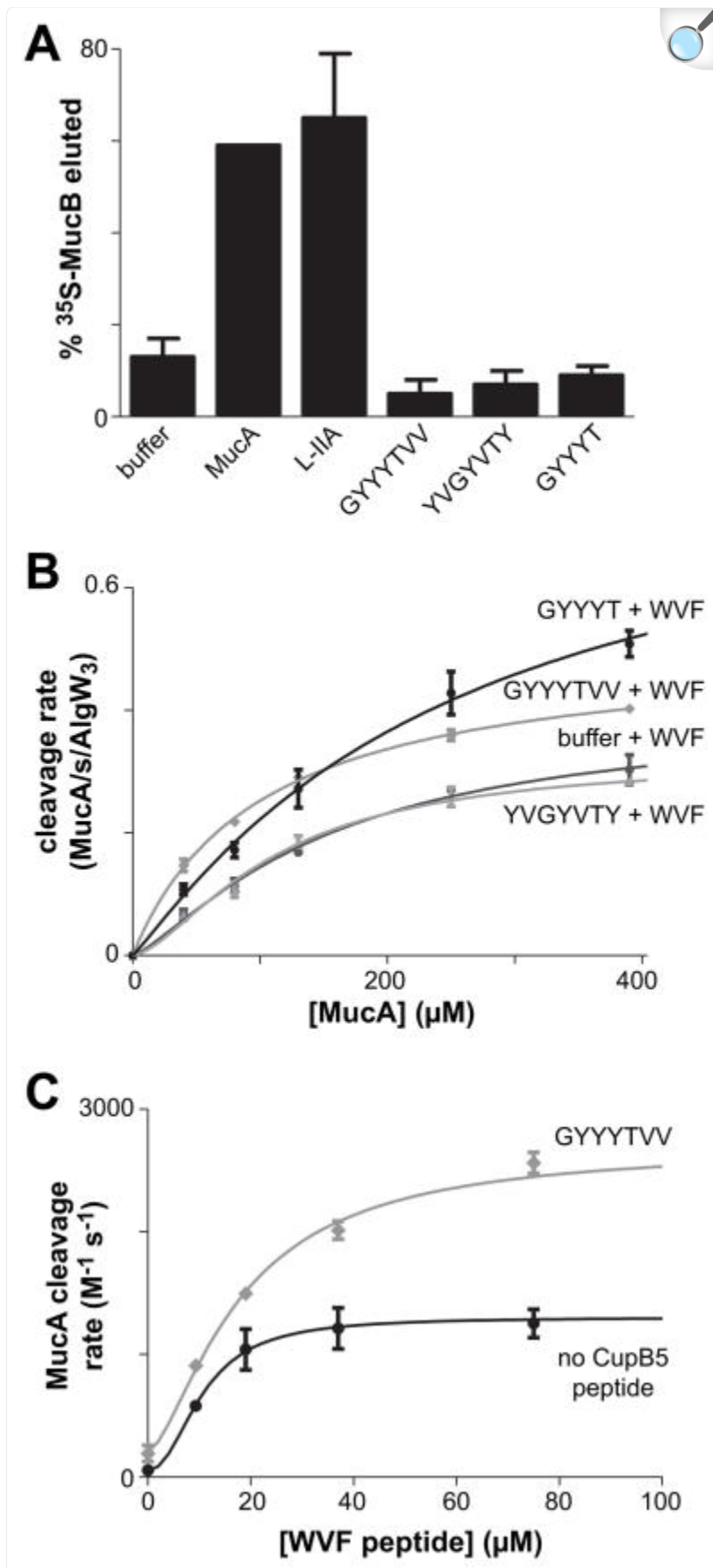
(A) Cleavage of  $^{35}S$ -labelled MucA (20  $\mu M$ ) by AlgW (0.5  $\mu M$  trimer) was assayed by the time-dependent release of acid-soluble peptides in the presence of either the OMP-like WVF peptide (400  $\mu M$ ), the wild-type GYYT or GYYTVV CupB5 peptides (400  $\mu M$ ), the sequence-scrambled YVGYYTY peptide (400  $\mu M$ ), or a buffer control. (B) Second-order rate constants for MucA cleavage were determined by incubating  $^{35}S$ -labelled MucA (20  $\mu M$ ) and AlgW (0.5  $\mu M$  trimer) with different concentrations of WVF or CupB5 peptides and measuring the time-dependent increase in acid-soluble products. Rates were divided by the MucA and AlgW concentrations to determine activity. Lines are fits to the equation  $activity = basal + stimulated / (1 + (K_{act}/[peptide])^h)$ . Values are averages  $\pm$  SEM ( $n=2$ ). (C) Second-order rate constants for cleavage of  $^{35}S$ -MucA (20  $\mu M$ ) by AlgW (0.5  $\mu M$  trimer) were determined as described in panel B in the presence of different combinations of WVF peptide (110  $\mu M$ ), MucB (25  $\mu M$  dimer), and wild-type or scrambled CupB5 peptides

(405  $\mu\text{M}$ ). Values are averages of two or more independent trials  $\pm$  SEM. (D) Second-order rate constants for cleavage of  $^{35}\text{S}$ -MucA (20  $\mu\text{M}$ ) by AlgW (0.5  $\mu\text{M}$  trimer) and WVF peptide (150  $\mu\text{M}$ ) were determined in the presence of increasing concentrations of the GYYTY, GYYTYTVV, or sequence-scrambled YVGYVTY peptides. Lines are fits to the Hill equation:  $\text{rate} = \text{basal} + V_{\text{max}}/(1 + (K_s/[\text{peptide}])^h)$ . For GYYTY,  $K_s$  was  $55 \pm 13$   $\mu\text{M}$  and  $h$  was  $2.1 \pm 0.9$ . For GYYTYTVV,  $K_s$  was  $143 \pm 31$   $\mu\text{M}$  and  $h$  was  $3.5 \pm 2.2$ .

As expected from previous studies ([Cezairliyan and Sauer, 2009](#)), addition of MucB suppressed cleavage of  $^{35}\text{S}$ -MucA by WVF-activated AlgW ([Fig. 4C](#)). Moreover, addition of just the GYYTYTVV, YVGYVTY, or GYYTY CupB5 peptides did not result in AlgW cleavage of MucA when MucB was present ([Fig. 4C](#)). Next, we tested if the wild-type or scrambled CupB5 peptides could relieve MucB inhibition of AlgW cleavage of  $^{35}\text{S}$ -MucA in the presence of the WVF activating peptide. Importantly, titration of GYYTYTVV or GYYTY into reactions containing MucA, MucB, AlgW, and WVF peptide resulted in a dose-dependent increase in the rate of MucA cleavage ([Fig. 4D](#)). Almost no increase in MucA cleavage was observed with the scrambled YVGYVTY peptide ([Fig. 4D](#)), demonstrating sequence specificity. Interestingly, activation of MucA cleavage by GYYTY occurred at lower peptide concentrations and was less cooperative as judged by the Hill constant than activation by the GYYTYTVV peptide ([Fig. 4D](#)). Thus, the C-terminal valine residues of GYYTYTVV are not necessary for activation of MucA cleavage in the presence of MucB *in vitro*. Although the GYYTYTVV and GYYTY peptides activated AlgW cleavage of MucA to similar levels in the presence of WVF peptide and MucB, these levels were approximately one-third of the MucA cleavage rate observed with WVF and AlgW alone (compare [Figs. 4B and 4D](#)). We conclude that the CupB5 peptides can partially relieve MucB inhibition of WVF-activated AlgW cleavage of MucA.

Lipopolysaccharides bind MucB and competitively displace MucA ([Lima et al., 2013](#)). Because MucB normally binds peptide sequences in MucA, it seemed plausible that CupB5 peptides might also compete for MucA binding to MucB. We tested this possibility by assaying the ability of the CupB5 peptides to elute  $^{35}\text{S}$ -MucB from MucA that had been covalently bound to agarose resin ([Fig. 5A](#)). In this assay, the L-IIA lipopolysaccharide fragment and free MucA efficiently competed with resin-bound MucA for MucB binding. However, incubation of  $^{35}\text{S}$ -MucB•MucA resin with the GYYTYTVV, YVGYVTY, or GYYTY peptides resulted in no more  $^{35}\text{S}$ -MucB release than observed in the buffer control ([Fig. 5A](#)). This result strongly suggests that the CupB5 peptides do not relieve MucB inhibition by directly competing for its binding to MucA. Binding of the L-IIA lipopolysaccharide fragment converts MucB dimers to tetramers ([Lima et al., 2013](#)). By contrast, incubation of MucB with GYYTYTVV peptide resulted in no substantial change in elution during gel-filtration chromatography ([Fig. S2](#)).

Fig. 5. CupB5 peptides do not compete with MucB for MucA binding but stimulate WVF-activated AlgW cleavage of MucA.



(A) Release of  $^{35}\text{S}$ -MucB from MucA-agarose following incubation with buffer, MucA (155  $\mu\text{M}$ ), L-IIA (20 mg/mL), or CupB5 peptides ( $\sim 300\text{ }\mu\text{M}$ ). For the CupB5 peptides, values are averages  $\pm$  SEM ( $n=3$ ). For L-IIA, the value is the average  $\pm$  SEM ( $n=2$ ). (B) Rates of cleavage by AlgW (0.5  $\mu\text{M}$  trimer) and WVF peptide (150  $\mu\text{M}$ ) were measured at different concentrations of  $^{35}\text{S}$ -MucA in the presence or absence of CupB5 peptides (300  $\mu\text{M}$ ). Values are averages  $\pm$  SEM ( $n=2$ ) and were fit to the Hill form of the Michaelis-Menten equation,  $\text{rate} = V_{\text{max}}/(1 + (K_{\text{M}}/[\text{substrate}])^h)$ . (C) Rates of cleavage of  $^{35}\text{S}$ -MucA (50  $\mu\text{M}$ ) by AlgW (0.5  $\mu\text{M}$  trimer) were assayed at different concentrations of WVF peptide in the presence or absence of CupB5 peptides (300  $\mu\text{M}$ ). Values are averages  $\pm$  SEM ( $n=2$ ) and were fit to the equation  $\text{rate} = \text{basal} + V_{\text{max}}/(1 + (K_{\text{act}}/[\text{peptide}])^h)$ . Without CupB5 peptide,  $h$  was  $2.2 \pm 1$ ,  $K_{\text{act}}$  was  $11 \pm 2\text{ }\mu\text{M}$ , and  $V_{\text{max}}$  was  $1250 \pm 150\text{ M}^{-1}\text{ s}^{-1}$ . With GYYYYTVV peptide,  $h$  was  $1.5 \pm 0.8$ ,  $K_{\text{act}}$  was  $18 \pm 7\text{ }\mu\text{M}$ , and  $V_{\text{max}}$  was  $2490 \pm 490\text{ M}^{-1}\text{ s}^{-1}$ .

Because CupB5 peptides did not strongly activate AlgW cleavage of MucA in the absence of the WVF peptide or release MucA from MucB, we speculated that they might act in concert with the WVF peptide to allow AlgW to bind and degrade MucA more efficiently and thus to compete better with MucB for the MucA substrate. This model predicts that MucA should be degraded at a faster rate in the presence of both the WVF and CupB5 peptides. Indeed, when we assayed the rate of cleavage of different concentrations of  $^{35}\text{S}$ -MucA by AlgW, faster cleavage was observed when both the WVF and wild-type CupB5 peptides were present compared to only the WVF peptide or the WVF peptide and scrambled CupB5 control peptide ([Fig. 5B](#)). We also measured the rate of AlgW cleavage of a fixed concentration of MucA in the presence of increasing concentrations of WVF peptide with or without additional GYYYYTVV peptide ([Fig. 5C](#)). When GYYYYTVV peptide was present, the maximum rate of MucA cleavage increased almost two-fold. We conclude that the CupB5 peptides and WVF peptide act synergistically to enhance AlgW cleavage of MucA, allowing better competition with MucB.

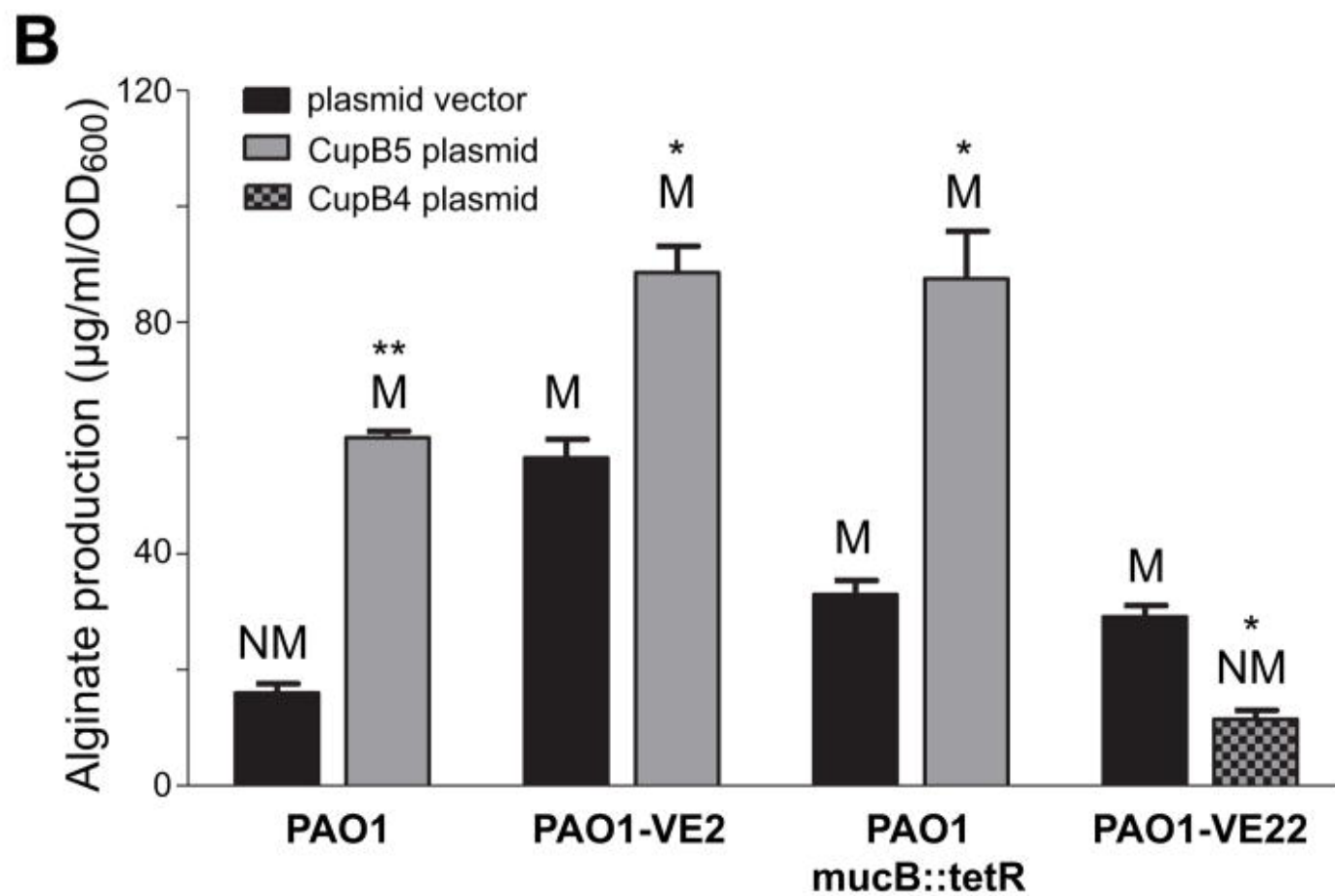
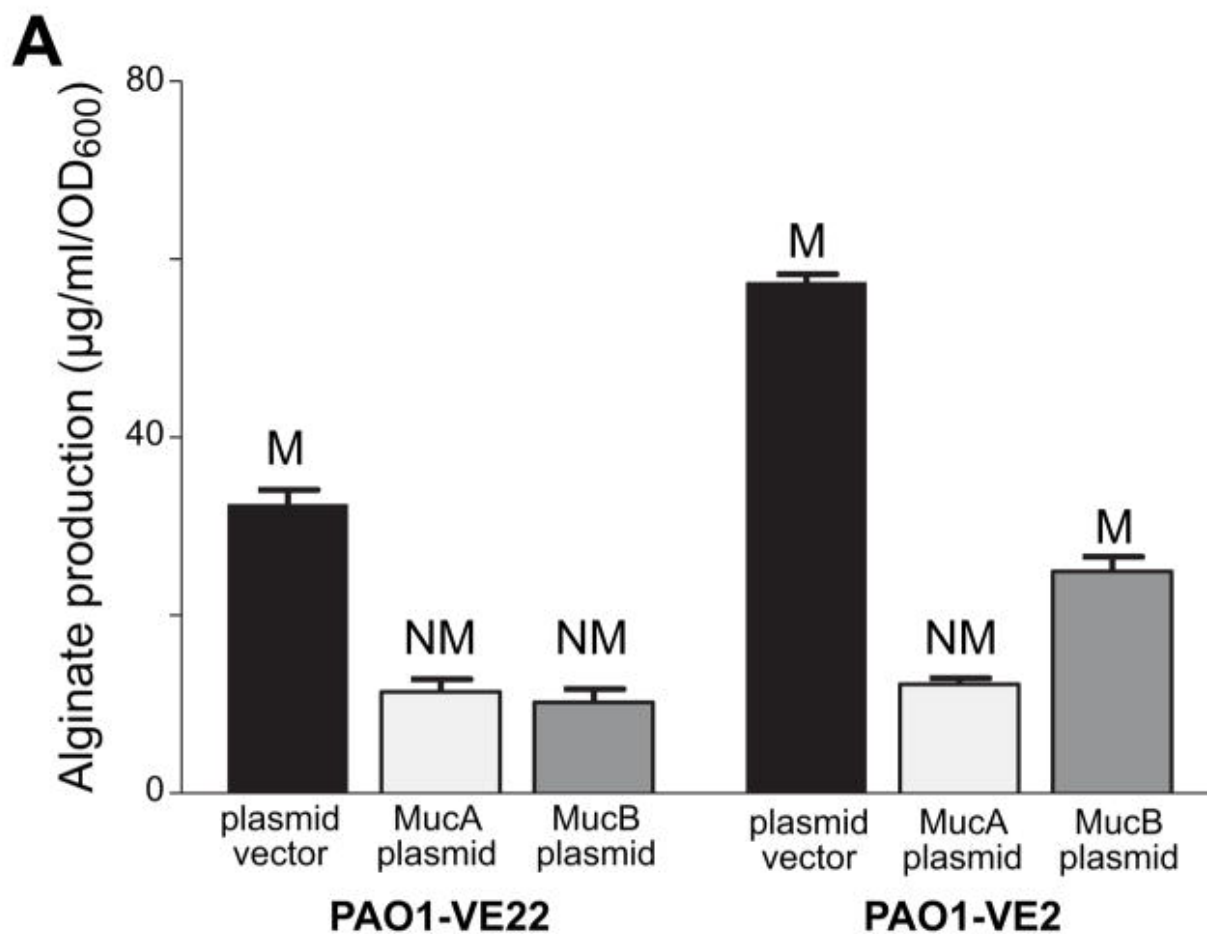
In *E. coli*, RseA, RseB, and DegS are homologs of MucA, MucB, and AlgW. A YYF peptide efficiently activates DegS cleavage of RseA in the absence of RseB or in the presence of both RseB and lipopolysaccharides ([Walsh et al., 2003](#); [Cezairliyan and Sauer, 2007](#); [Lima et al., 2013](#)). The CupB5 peptides did not substantially stimulate cleavage of  $^{35}\text{S}$ -RseA by YYF-activated DegS in the presence of RseB ([Fig. S3](#)), suggesting that they do not interact with DegS. This result is not surprising, as DegS and AlgW share only 44% sequence homology.

## Suppressing and enhancing alginate production

If CupB5 allows AlgW to compete more efficiently with MucB for MucA binding and cleavage, then overproduction of MucB in strain PAO1-VE22 would be expected to decrease alginate production. Indeed, plasmid-mediated MucB overproduction had this effect, as did plasmid-mediated overproduction of MucA ([Fig. 6A](#)). The latter result suggests that with enough additional uncleaved MucA, AlgU is efficiently inhibited even when more MucA is degraded than usual. Even the CupB5-induced elevated rate of AlgW cleavage of MucA in this strain must be too slow to free enough

AlgU to fully enhance transcription of alginate biosynthesis genes.

Fig. 6. Inducing and suppressing alginate production in *P. aeruginosa* strains.





(A) Strains overproducing CupB5 (PAO1-VE22) or MucE (PAO1-VE2) were transformed with plasmid HERD20T or variants overexpressing MucA or MucB, grown at 37°C for 24 h on PIA plates supplemented with 0.1% L-arabinose, and alginate production and colony phenotypes (M, mucoid; NM, nonmucoid) were assayed. (B) Alginate production and mucoid phenotypes were assayed in strains PAO1, PAO1-VE2 (*mucE* overexpressed), PAO1 *mucB::tetR* (*mucB* inactivated), and PAO1-VE22 (*cupB5* overexpressed) transformed with the HERD20T plasmid vector or variants expressing HA-CupB4-H<sub>6</sub> or HA-CupB5-H<sub>6</sub>. Values are averages ± SEM (n=3). Statistical significance was determined using the Student's *t*-test (\*P<0.05; \*\*<0.005).

Plasmid-expressed MucB or MucA also decreased alginate production in PAO1-VE2, a strain expressing higher MucE levels ([Fig. 6A](#)). The rate of AlgW cleavage of MucA in PAO1-VE2 is apparently too low to fully degrade the excess MucA, resulting in greater inhibition of AlgU and reduced alginate production. Excess MucB presumably outstrips the endogenous anti-MucB signals in PAO1-VE2 and mass action results in enhanced binding to MucA, reducing the rate of MucA cleavage by activated AlgW.

To determine how simultaneous overexpression of MucE and CupB5 affected alginate levels, we transformed PAO1-VE2 with a plasmid expressing HA-CupB5-H<sub>6</sub>. Notably, alginate levels in this strain were higher than in the empty-vector control ([Fig. 6B](#)), suggesting that activation of AlgW through MucE and through CupB5 occur synergistically. Our biochemical results predict that MucB should not be genetically required for CupB5-mediated pathway activation. To test this prediction, we used a transposon-insertion strain, PAO1-*mucB::tet<sup>R</sup>*, which displayed an elevated level of basal alginate production and mucoid phenotype ([Fig. 6B](#)). When we transformed PAO1-*mucB::tet<sup>R</sup>* with a plasmid expressing HA-CupB5-H<sub>6</sub>, alginate production increased compared to the empty-vector control ([Fig. 6B](#)), indicating that CupB5 overexpression induces pathway activation by a MucB-independent mechanism. Finally, we tested if expression of the CupB4 chaperone, which should facilitate export of CupB5 rather than its periplasmic accumulation, would decrease alginate production. Indeed, when we transformed PAO1-VE22 with a plasmid expressing HA-CupB4-H<sub>6</sub>, alginate production was suppressed and the mucoid phenotype of the parent strain was reversed ([Fig. 6B](#)). This result supports a model in which interactions between accumulated periplasmic CupB5 and periplasmic AlgW leads to induction of alginate production and mucoidy.

## Discussion

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Based on results from a transposon screen in *P. aeruginosa*, we discovered that overexpression of CupB5, a protein normally secreted through the outer membrane by a chaperone-usher system, activates alginate production and results in mucoid colonies. CupB5-mediated mucoidy depends on known components of the AlgU stress-response pathway,

including the AlgU transcription factor and the AlgW, MucP, and ClpXP proteases. Peptides from a region of CupB5 important for elevated alginate production *in vivo* stimulate cleavage of MucA by OMP-activated AlgW *in vitro* and partially relieve MucB inhibition of AlgW cleavage of MucA. Overexpression of either MucB or MucA in strains that overexpress CupB5 also reduces alginate production, supporting a model in which excess periplasmic CupB5 stimulates proteolytic cleavage of MucA by AlgW.

We found that expression of HA-CupB5<sup>1-491</sup>-H<sub>6</sub> in PAO1 increased alginate production *in vivo* and demonstrated that a GYYTTVV peptide (corresponding to CupB5 residues 485–491) antagonized MucB inhibition *in vitro*. A peptide with the same composition as GYYTTVV but a scrambled sequence did not inhibit MucB. However, we also found that expression of HA-CupB5<sup>1-489</sup>-H<sub>6</sub> failed to increase alginate production *in vivo*, whereas a GYYTYT peptide (corresponding to CupB5 residues 485–489) did antagonize MucB inhibition *in vitro*. This discrepancy between results *in vivo* and *in vitro* could arise because the GYYTYT peptide in HA-CupB5<sup>1-489</sup>-H<sub>6</sub> is altered by proteolysis or is masked by tertiary structure, aggregation, or binding to another cellular component. We also note that expression of full-length HA-CupB5-H<sub>6</sub> and truncated HA-CupB5<sup>1-585</sup>-H<sub>6</sub> resulted in somewhat higher alginate levels than expression of HA-CupB5<sup>1-491</sup>-H<sub>6</sub>. This variation could arise from differences in expression levels, cellular interactions, or possibly as a consequence of additional peptide sequences in the longer proteins that facilitate pathway activation.

We initially thought that CupB5 peptides might bind MucB and compete for binding to MucA in a manner analogous to LPS. However, neither GYYTTVV nor GYYTYT peptides compete with MucA-agarose for MucB binding and saturating concentrations of these peptides only partially relieve MucB inhibition *in vitro*. Moreover, unlike LPS, GYYTTVV did not convert MucB dimers into tetramers. By themselves, CupB5 peptides activate AlgW cleavage *in vitro* far less well than the OMP-like WVF peptide. However, CupB5 peptides stimulate MucA cleavage by WVF-activated AlgW, suggesting that OMP-like and CupB5 peptides bind to distinct sites in AlgW but have a common effect of stabilizing the proteolytically active enzyme.

The GYYTTVV sequence is conserved in CupB5 proteins from different strains of *P. aeruginosa* but not in CupB5 proteins from other *Pseudomonas* species ([Fig. S3](#)), and some *Pseudomonas* species have no CupB5 ortholog. Thus, CupB5 activation of AlgW is specific to *P. aeruginosa*. It remains to be determined if *P. aeruginosa* utilizes other proteins in this manner or if AlgW homologs in other bacteria are activated by more than one kind of peptide signal.

Activation of the AlgU pathway requires one molecular signal (C-terminal OMP peptides) to activate site-1 cleavage of MucA by the AlgW protease and a second molecular signal (LPS) to relieve MucB inhibition of this cleavage. Overproduction of CupB5 can partially bypass this second requirement by helping OMP-bound AlgW compete with MucB for MucA binding and proteolysis. Expression of CupB5 under non-stress conditions increases alginate production similarly to non-stress expression of MucE ([Qiu et al., 2007](#)). In these experiments, a signal that directly antagonizes MucB inhibition could arise indirectly as a consequence of other molecules whose concentrations increase as a consequence of CupB5 or MucE expression or might always be present at some level in the periplasm. For

example, some level of unassembled OMPs and LPS is probably always present in the periplasm. Notably, however, simultaneous expression of CupB5 and MucE results in higher levels of alginate, indicating that these signals activate the alginate pathway through synergistic mechanisms

Although CupB5 peptides, OMP peptides, and LPS molecules function by different mechanisms to activate AlgW or relieve MucB inhibition, the molecular logic that gives rise to all three signals is similar. For example, stresses that compromise the transport to and outer-membrane assembly of OMPs and LPS, or that slow usher-mediated CupB5 secretion, would all lead to the periplasmic accumulation of these molecules and activation of the AlgU pathway. Indeed, we found that alginate production was suppressed in strains that overproduced the CupB4 chaperone, allowing export of CupB5. Certain stresses might only activate AlgW through OMP signals, whereas others require an enhanced response via CupB5 or possibly other proteins secreted through the outer membrane. Clearly, however, the AlgU system integrates multiple signals, each reporting on potential dysfunction in outer-membrane assembly, structure, and/or secretion. Dysfunction in just one pathway should give rise to a buffered AlgU response, whereas dysfunction affecting multiple pathways would elicit a robust AlgU response.

## Experimental procedures

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### Bacteria strains, plasmids, and growth conditions

Bacteria strains and plasmids used in this study are shown in [Table S2](#). *Escherichia coli* strains were grown at 37°C in Lennox broth (LB) or LB agar. *P. aeruginosa* strains were grown at 37°C in LB, *Pseudomonas* isolation broth (PIB, Alpha Bioscience) or on *Pseudomonas* isolation agar (PIA) plates (Difco). When required, the concentration of carbenicillin, tetracycline or gentamicin added to LB broth or plates was 100 µg ml<sup>-1</sup>, 20 µg ml<sup>-1</sup>, or 15 µg ml<sup>-1</sup>, respectively, and 300 µg ml<sup>-1</sup>, 150 µg ml<sup>-1</sup>, or 200 µg ml<sup>-1</sup> when added to PIB or PIA plates, respectively.

### Transposon mutagenesis

Transposon mutagenesis was carried out via biparental conjugations using *E. coli* SM10 λpir carrying plasmid pFAC as the donor strain ([Wong and Mekalanos, 2000](#)) and PAO1 as the recipient strain. After incubating PAO1 and *E. coli* cells on LB agar plates at 37°C for 6 h, bacteria were collected, re-suspended in PBS and then plated onto PIA supplemented with gentamicin (200 µg ml<sup>-1</sup>). Mucoid colonies were identified. The chromosomal DNA of mucoid mutants was isolated using the QIAamp genomic DNA Extraction kit (Qiagen, USA). Approximately, 2 µg of DNA was digested with *Sall* overnight at 37°C, followed by purification and self-ligation using Fast-Link DNA ligase (Epicentre, USA). The circular closed DNA was used as a template for inverse PCR using GM3OUT and GM5OUT primers ([Qiu et al., 2008b](#)). The PCR products were purified and sequenced. Finally, Southern-blot hybridization was also conducted to monitor the copy number of transposon insertions using the Gm<sup>r</sup> gene as the probe ([Head and Yu, 2004](#)).

## Genetic construction, transformation and conjugation

All DNA cloning was performed using PCR products digested with appropriate restriction enzymes and ligated into the *E. coli* to *Pseudomonas* shuttle vector pHERD20T ([Qiu et al., 2008a](#)). All constructs of pHERD20T were sequenced to verify the correct DNA sequences. DNA sequencing was carried out by the Marshall University Genomics Core Facility. The primer sequences used for PCR cloning will be provided upon request. The transformation of One Shot TOP10 (Invitrogen, USA) was performed according to the supplier's instruction. The transfer of pHERD20T and its derivatives was performed via triparental conjugation using the helper plasmid pRK2013 ([Figurski and Helinski, 1979](#)).

## Protein preparation and Western blotting

For extraction of total cellular protein, PeriPreps™ Periplasting Kit (Epicentre, USA) was used. Bacteria were inoculated in PIB medium and incubated in shake culture for several hours, until the OD<sub>600</sub> was ~0.6. At this point 0.1% L-arabinose was added to induce protein expression. Two hours after induction, bacteria were collected for cell lysis. The protein concentration was measured using Bio-Rad Dc protein assay reagents (Bio-Rad). Equivalent proteins were mixed with 2×sample loading buffer and analyzed on 10–20% pre-cast SDS-PAGE gels (Bio-Rad). Proteins were then transferred to a PVDF membrane (GE) by electro-blotting for immunodetection. A primary antibody, rat Anti-HA (Roche, USA), was used at a dilution of 1:5000. Goat anti-rat immunoglobulin G (heavy and light chains) conjugated with horseradish peroxidase (Pierce, USA) diluted 1: 5000 was used as the secondary antibody. Finally, the proteins on the membrane were visualized using the Amersham ECL kit (GE, USA).

## Alginate assay

*P. aeruginosa* strains were grown at 37°C on triplicate PIA plates for 24 h. Bacteria were collected, suspended in PBS, and the OD<sub>600</sub> was measured and adjusted to ~0.5 by addition of PBS. The suspensions were analyzed for the amount of uronic acid in comparison with a standard curve made with D-mannuronic acid lactone (Sigma-Aldrich, USA) as described ([Damron et al., 2009](#)).

## β-galactosidase assay

*Pseudomonas* strains carrying the *lacZ* vector pLP170 containing *P<sub>algU</sub>*, and *P<sub>algD</sub>* were cultured on PIA plates. After 24 h, bacteria were harvested, resuspended in PBS, and OD<sub>600</sub> was measured and adjusted to ~0.3. Cells were then permeabilized using toluene, and β-galactosidase activity was measured at OD<sub>420</sub> and OD<sub>550</sub>. The results in Miller Units were calculated according to the follow formula: Miller Units=1000×[OD 420–(1.75 × OD 550)]/[Reaction time (m) × Volume (ml) × OD 600] ([Miller, 1972](#)). The reported values represent an average of three independent experiments ± SEM.

## Proteins and peptides

Purifications of *P. aeruginosa* MucA, MucB and AlgW and *E. coli* RseA, RseB and DegS were performed as described after plasmid-mediated overexpression in *E. coli* strain X90(DE3) ([Sohn \*et al.\*, 2007](#); [Cezairliyan and Sauer, 2009](#)). Cultures containing 100 µg/mL ampicillin were grown to an OD600 of ~0.6, and protein expression was induced with 100 mM IPTG for two h before harvesting. Cells overexpressing MucB, AlgW, RseB or DegS were resuspended in native lysis buffer [*P. aeruginosa* proteins: 50 mM sodium phosphate (pH 8), 500 mM KCl, 20 mM imidazole; *E. coli* proteins: 50 mM sodium phosphate (pH 8), 300 mM NaCl, 10 mM imidazole] and lysed by sonication. Cells overexpressing MucA or RseA were resuspended in denaturing lysis buffer [50 mM sodium phosphate (pH 8), 8 M guanidine hydrochloride (pH 8)] and allowed to lyse at room temperature for 30 min. For all preparations, lysates were cleared by centrifugation and applied to Ni-NTA columns pre-equilibrated in native lysis buffer or denaturing lysis buffer. Columns were washed with lysis buffer and proteins eluted with elution buffer [*P. aeruginosa* proteins: 50 mM sodium phosphate (pH 8), 500 mM KCl, 500 mM imidazole; *E. coli* proteins: 50 mM sodium phosphate (pH 8), 300 mM NaCl, 300 mM imidazole]. Proteins were then dialyzed overnight against storage buffer [*P. aeruginosa* proteins: 50 mM sodium phosphate (pH 7.4), 200 mM KCl, 10% glycerol; *E. coli* proteins: 50 mM sodium phosphate (pH 8), 200 mM NaCl, 1 mM EDTA] and then again against fresh buffer before being stored frozen at  $-80^{\circ}\text{C}$ . Prior to dialysis and storage, MucB and RseB were chromatographed on a size-exclusion column and only fractions corresponding to the dimer were collected. To obtain radiolabeled  $^{35}\text{S}$ -MucA,  $^{35}\text{S}$ -RseA, or  $^{35}\text{S}$ -MucB, cells were grown in a defined rich medium (TekNova) lacking methionine and  $^{35}\text{S}$ -methionine was added upon induction. Purification of  $^{35}\text{S}$ -MucA and  $^{35}\text{S}$ -RseA then proceeded as described above for unlabeled MucA and RseA. Cells expressing  $^{35}\text{S}$ -MucB were lysed by the addition of lysozyme and three freeze-thaw cycles, and purification then proceeded as described above. All peptides were synthesized by GenScript and purified by reverse-phase HPLC using a C18 column. Purified products were lyophilized, stored at  $-20^{\circ}\text{C}$ , then resuspended in storage buffer and used immediately. The L-IIA LPS fragment was a gift from Santiago Lima (MIT) ([Lima \*et al.\*, 2013](#)).

## Cleavage assays

Assays were performed as described ([Sohn \*et al.\*, 2007](#); [Cezairliyan and Sauer, 2009](#)). Briefly, cleavage reactions were performed at  $25^{\circ}\text{C}$  in reaction buffer [*P. aeruginosa* proteins: 50 mM sodium phosphate (pH 7.4), 200 mM KCl, 10% glycerol; *E. coli* proteins: 150 mM sodium phosphate (pH 8.3), 380 mM NaCl, 10% glycerol, 4 mM EDTA]. Reactions were initiated by the addition of protease and quenched after different times by addition of cold 10% trichloroacetic acid. Following centrifugation, acid-soluble reaction products were quantified by scintillation counting and compared to total radioactivity from control samples. Cleavage activity for peptide-activation assays and experiments involving MucB are reported as a second-order rate constant calculated by dividing the rate of substrate cleavage by the substrate and enzyme concentrations. Activity at different substrate concentrations for the Michaelis-Menten curve was calculated by dividing the rate of substrate cleavage by the enzyme concentration.

## MucA-resin assay

Dry cyanogen-bromide activated Sepharose B4 resin (66 mg) was washed with 1 M HCl, equilibrated with coupling buffer [25 mM sodium phosphate (pH 8.3), 50 mM NaCl], and incubated with purified MucA (900 µg) for 1.5 hours. After removal of free protein, reactive groups on the resin were blocked by incubation in 0.5 M ethanolamine (pH 8.3) for 1 h. The MucA resin was washed, resuspended in coupling buffer, and stored at 4 °C. For assays, MucA resin was drained, incubated with a four-fold molar excess of <sup>35</sup>S-MucB, washed again, incubated with ligand for 30 min, added to a pre-wetted 96-well Multiscreen HTS-HV Filter Plate (Millipore) over a 96-well polypropylene receiving plate (Greiner), and centrifuged at 1500 × g for 1 min. Elution of <sup>35</sup>S-MucB was measured by scintillation counting and normalized by dividing by the total initial <sup>35</sup>S-MucB radioactivity.

## Supplementary Material

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Supp FigureS1-S3 & TableS1-S2

[NIHMS604546-supplement-Supp\\_FigureS1-S3\\_TableS1-S2.pdf](#) (587KB, pdf)

## Acknowledgments

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## Associated Data

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*This section collects any data citations, data availability statements, or supplementary materials included in this article.*

## Supplementary Materials

Supp FigureS1-S3 & TableS1-S2

[NIHMS604546-supplement-Supp\\_FigureS1-S3\\_\\_TableS1-S2.pdf](#) (587KB, pdf)